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PRINCIPAL INVESTIGATOR: Shu-Yuan Chiang, Ph.D.

CONTRACTING ORGANIZATION: Health Research Incorporated
Buffalo, New York 14263

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Buffalo, New York 14263

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Stephanie Chung June 25, 88

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Abstract

Abnormally expressed ESX and AP-2 have been found in certain breast cancer cell lines that are associated with the overexpression of HER2/*neu* gene. Amplification and overexpression of HER2/*neu* is found in 20-30% of primary breast cancers and is correlated with a poor prognosis. In this study, polyamides, minor groove binding compounds, were designed to target the ESX binding site on the HER2/neu promoter to interfere with the gene expression. The effects of polyamides to inhibit the binding of ESX and DNA and associated gene expression were compared with that of distamycin. The results revealed that polyamides were more effective than distamycin to inhibit the ESX-DNA complex formation (25-200 fold). Similarly, in a cell free transcription, polyamides inhibited gene expression from the HER2/*neu* promoter more strongly than distamycin. In addition, *in vitro* transcription time course assay indicated that polyamides constantly associated with DNA to inhibit transcription. In contrast, inhibition of transcription by distamycin was increased relative to the time of incubation. In a conclusion, sequence specific designed polyamides for selected transcription factors are potent inhibitors of transcription factor-DNA complex and transcription. This information can be utilized to future improve drug specificity and effectiveness as abnormal transcription inhibitors.

Introduction

Consideration of transcription factors (TFs) as targets for antitumor agents is based upon the recognition that abnormal regulation of gene expression plays an important role in cancer (1,2) The first step in the regulation of gene expression requires TF binding to the promoter region (3, 4). Despite advancements in understanding mechanisms by which TFs regulate gene expression at the molecular level, utilization of this information to design inhibitors has lagged. The ability to preferentially block gene expression by interfering with specific TF-DNA complexes could be a powerful tool for elucidating how aberrant gene expression contributes to neoplastic phenotypes.

One approach to inhibit complexes between TFs and their consensus DNA binding domains is to target DNA binding drugs such as minor groove binders and intercalators to the TF consensus DNA binding site (5,6). In general, matching the drug DNA binding motif to that of the TF-DNA complex results in enhancement of its inhibition activity (5, 7, 8). Interestingly, DNA minor groove binding drugs directed to TF DNA binding domains, are effective at inhibiting TF-DNA complexes that utilize only major groove contacts, probably due to groove distortions (9, 10). More recently, studies have shown that specially designed DNA minor groove binding drugs, microgonotropens, which have the ability not only to bind the DNA minor groove but also to interact with the DNA backbone of the major groove, are very effective at inhibiting complexes between E2F1 and its DNA binding site within the dihydrofolate reductase gene promoter (11-13).

A number of drugs which interfere with the binding of TFs to their consensus DNA binding sites within gene promoters also inhibit gene expression. Drugs like mithramycin, a GC binding DNA minor groove binder, is a potent inhibitor of c-myc regulated gene expression from the GC rich P1 promoter (7, 14). Similarly, drugs such as the DNA intercalator mitoxantrone and the minor groove binding distamycin, both of which can inhibit the binding of E2F1 to its promoter sequence contained within the dihydrofolate reductase gene, are strong inhibitors of its expression (15). Distamycin is also known to inhibit basal in vitro transcription by interfering with the association of TBP with its promoter (16).

All of the drugs studied so far are based upon a general sequence preference for the TF DNA binding site rather than being specifically designed to target elements within the consensus binding region. It is the purpose of this study to explore the development of a new class of novel DNA minor groove binding drugs, polyamides, as inhibitors targeted at the HER2/*neu* promoter (17). Polyamides (PAs) represent a significant advancement in drug design in that they can achieve a remarkable degree of sequence recognition and can tightly associate with DNA (18-20). PAs that contain N-methylimidazole (Im) and N-methylpyrrole (Py) can bind side by side in an anti-parallel fashion that specifically recognizes a GC base pair (Im-Py) or a CG pair (Py-Im), by substituting a hydroxypyrrole (hP) for pyrrole (hP has a greater affinity for T than Py, while the converse is true for A), polyamides can also distinguish between TA (hP-Py) and AT (Py-hP) bases (17, 21). Recently, one of these compounds specifically designed to interfere with TFIIIA binding to promoter elements, was shown to be a potent and specific inhibitor of TFIIIA regulation of the 5S RNA gene (22).

PAs offer the prospect of being highly specific at inhibiting a designated TF-DNA complex. To test this notion, a member of the Ets family of TFs ESX, which has been found to associate with the expression of HER2/*neu* gene, was chosen as a target. ESX binding to the HER2/*neu* gene promoter results in a deregulation and overexpression of this growth factor receptor proto-oncogene which is linked to human breast cancer (23-25). In this study three PAs were synthesized that recognized varying DNA sequences around the ESX binding site (Figure 1). A comparison was made between the ability of these three drugs as well as the classical DNA minor groove binding drug distamycin to inhibit the binding of ESX to its consensus DNA binding site. In addition, we examined the ability of the drugs to inhibit the TF AP-2, which binds to a GC rich region adjacent to the ESX binding site and is known to be involved in the abnormal expression of HER2/*neu*. An *in vitro* transcription assay was used to evaluate the ability of the drugs to interfere with gene transcription from the Her2/*neu* promoter.

Materials and Methods

Cell Culture. SKBR-3 cells were purchased from ATCC (Rockville, MD). Cells were grown in McCoy's 5a medium (GIBCO, Grand Island, NY) with 10% fetal bovine serum, and cultured at 37 °C with 5% CO_2.

Nuclear Extract preparation. SKBR-3 cells that were grown to subconfluence were rinsed twice with Phosphate-buffered saline, scraped, and collected by centrifugation at 1,200 rpm for 5 min, 4 °C (Sorvall RT6000, Newtown, CT). The following steps were performed at 4 °C. Cell pellets were suspended in five times the packed cell volume in buffer A (containing 10 mM Hepes-KOH [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.75 mM spermidine, 0.15 mM spermine, and 1 mM dithiothreitol), followed by centrifugation at 1,200 rpm for 5 min. The pellet was then resuspended in five times the pellet volume in buffer A, kept on ice for 8 min, and homogenized with 10 strokes using a Dounce homogenizer (tight pestle). The homogenate (~ 95% lysed cells) was centrifuged at 15,000 rpm for ~ 1 min, (JA-17 rotor, JA-21 centrifuge; Beckman, Palo Alto, CA). The pellet was resuspended in buffer B with 20 mM Hepes-KOH (pH 7.9), 20% glycerol, 0.2 mM EDTA, 0.2 mM EGTA, 0.75 mM spermidine, 0.15 mM spermine, 2 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride followed by drop addition of an equal volume of buffer B that included 0.75 M NaCl. After rocking for 20 min, supernatant was collected by centrifugation at 47,500 rpm for 45 min (SW-55 rotor, Beckman), and dialyzed against > 100-fold buffer C (20 mM Hepes-KOH [pH 7.9], 20% glycerol, 100mM KCl, 0.2 mM EDTA, 0.2 mM EGTA, 12.5 mM $MgCl_2$, 2 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride) for 3h. Precipitated debris was removed by centrifugation at 15,000 rpm (JA-21 centrifuge, JA-17 rotor, Beckman) and the protein content of the nuclear extract was quantitated using the Bio-Rad protein assay.

Proteins and Antibodies. Bacterial-expressed ESX protein was prepared as described [Chang et al., 1997]. Briefly, full-length ESX cDNA was cloned into a pRSET his-tag expression plasmid (NheI-HindIII; Invitrogen). Expression of ESX protein was processed by transformation of bacterial cells (BL21[DE3] pLysS competent bacterial cells; Stratagene, La Jolla, CA), and induced by addition of IPTG. His-tagged ESX protein was purified using nickel-chelate affinity

chromatography as recommended by the manufacturer (Quiagen Inc., Chatsworth, CA). AP-2 protein was purchased from Promega Co. (Madison, WI). Antibodies against AP-2 was purchased from Santa Cruz Co. (La Jolla, CA), and antibodies against ESX was prepared from Dr. Benz's laboratory.

Oligonulceotides. A 34-mer oligonucleotide (oligo) containing the ESX protein binding site (derived from HER2 gene promoter; TA5-oligo) and its complementary strand were synthesized from the Biopolymer Facility (RPCI, Buffalo, NY) (sequence is shown in Fig. 1A). Oligos were gel-purified, annealed, and end-labeled with γ-^{32}P-[ATP] using T4-polynucleotide kinase (New England BioLabs, Beverly, MA) as described previously (8). ^{32}P-labeled 5' end -overhanging doubled-stranded oligo was used as the probe in gel mobility shift assays

Mobility Shift Assay. Demonstration of proteins binding to their consensus binding sequences was performed by gel mobility shift assay. In general, proteins at the indicated concentrations and 1 nM ^{32}P-labeled oligo were incubated in a reaction buffer containing 25 mM Tris (pH 7.5), 30 mM KCl, 5% glycerol, 0.1% NP-40, bovine serum albumin (100 μg/ml), and 1 mM dithiothreitol. After incubation at room temperature for 30 min, samples were loaded onto 5% native polyacrylamide gels, running with TBE buffer (44.5 mM Tris-base, 44.5 mM boric acid, 1 mM EDTA, pH 8.3). Then, the gel was dried and exposed to Kodak film. The protein-DNA complex was quantitated using a computing laser densitometer (Molecular Dynamics, Sunnyvale, CA). The amount of protein which could complex with >90% of ^{32}P-labeled oligo was used for most experiments. Identification of protein-DNA complexes was confirmed by adding specific antibodies against proteins to the reaction.

Drug Assay. The ability of polyamides to interfere with the formation of ESX-DNA complex was examined by a gel mobility shift assay. Assays were performed to determine the ability of polyamides to inhibit ESX-DNA complex under equilibrium conditions. Experiments were set up to incubate polyamides with ^{32}P-labeled oligo at room temperature for 30 min prior to the addition of ESX protein (standard), or to complex ESX protein with the probe before adding polyamides (reverse). The inhibition of ESX-DNA complex formation by PAs was measured by comparing

drug-treated with non drug-treated samples. Investigation of the ability of PAs to inhibit the AP-2-DNA complex was carried out in a similar manner. IC_{50} (concentration of drug required for 50% inhibition of protein-DNA complex formation) was used to express the activity of polyamides. Drug concentrations for IC_{50} were also expressed as r values (the molar ratio of drug to DNA base pairs).

***In Vitro* Transcription**. *In vitro* transcription was performed in a buffer containing 12 mM Hepes-KOH (pH 7.9), 60 mM KCl, 7.5 mM $MgCl_2$, 12 % glycerol, 0.12 mM EDTA, 0.12 mM EGTA, 1.2 mM DTT, and 0.6 mM PMSF. CsCl-purified plasmid DNA (RO6), composed of an insert DNA fragment from the HER2 promoter in the vector pCDNA3-Luc (Invitrogen, Carlsbad, CA), was linearizd with restriction enzyme *Sph*I (New England BioLabs, Beverly, MA) and used as a DNA template. In a 25 μL reaction, 1 μg of *Sph*I-digested DNA, nuclear extracts, 0.5 μL of each nucleotide (20 mM of ATP, GTP, UTP, and 100 μM CTP), 10 μCi of α-^{32}P-[CTP] (800 ci/mmole; NEN, Boston, MA), 1 μL of RNAsin (40 U/μL; Boerhringer Mannheim, Indianapolis, IN), and 1.4 μL of EDTA (2.5 mM) were incubated at 30 °C for 60 min. The reaction was stopped by adding 325 μL of 10 mM Tris-base (pH 8.0), 7 M urea, 350 mM NaCl, 1% SDS, and 50 ~ 100 μg tRNA, followed by phenol-chloroform-isoamyl alcohol extraction and ethanol precipitation. Samples were resuspended in formamide-loading dye and heated at 90~95 °C for ≥ 1 min before loading onto a 4%, 7 M urea-polyacrylamide gel. The ^{32}P signal from a dried gel was visualized using a Phosphorimager screen and quantitated with a computing laser densitometer (Molecular Dynamics, Sunnyvale, CA). The reaction was optimized by titration of nuclear extracts against 1 μg of DNA template in which a saturated condition was used for drug studies.

Effects of Polyamides on *In Vitro* Transcription. The ability of polyamides to inhibit *in vitro* transcription was analyzed by in ways. (i) One μg DNA template was incubated with polyamides at the indicated concentrations in a total volume of 10 μL for 30 min prior to the addition of nuclear extracts and nucleotides (standard). (ii) Pre-incubation of nuclear extracts and DNA template for 15 min was followed by addition of polyamides for another 30 min in a total volume of 10 μL and then nucleotides were added (reverse). All experiments were performed at 30 °C and the transcription reaction was allowed to proceed for 60 min. Percentage of inhibition of

transcription was measured by comparing samples treated with polyamides to an untreated control. IC_{50} and r values were used to express the activity of polyamides. T3 transcript (250 bases; Promega Co., Madison, WI) was used as an internal control.

Time Course of Transcription in the Presence of Drugs. The pattern of transcription from the HER2 promoter in the presence of PA-2E and distamycin was examined using a time course assay as described previously (15). Experimentally, DNA template incubated with SKBR-3 nuclear extracts for 15 min at 30 °C followed by addition of drug (concentration used to inhibit about 30 ~ 50% of transcription) and the nucleotides (ATP, CTP, GTP, UTP), and the reaction was stopped at different time points (i.e., 5, 10, 30 and 60 min). Transcripts from individual samples were normalized to an internal control and expressed as a percentage of relative transcription of an untreated control.

Results

Comparison of Effects of A Polyamide and Distamycin on ESX-DNA Complex Formation. The activity of newly designed DNA-binding agents PAs, targeting DNA sequences adjacent to the binding site of ESX, was compared with distamycin, an AT-preferential DNA minor groove binding agent, for their ability to interfere with the binding of ESX to its consensus DNA binding site. Both drugs would target a similar portion (site 2) of the ESX DNA binding domain. A gel mobility shift assay was utilized to examine the activities of drugs on ESX-DNA complex. Incubation of PA-2E and DNA followed by the addition of ESX resulted in a concentration-dependent inhibition of formation of the ESX-DNA complex. Ten nM PA-2E inhibited complex formation up to 95% while as little as 1 nM resulted in a detectable decrease of the complex (Figure 2A, lanes 2-4). There was no effect on the complex formation at a drug concentration of 0.1 nM (Figure 2A, lane 6). The pattern of inhibition of ESX-DNA complex formation by distamycin is similar to that by PA-2E but significantly higher drug concentration is required. Distamycin at 2000 nM diminished complex by ~ 95% (Figure 2 B, lane 3). While PA-2E at 100 nM inhibited the ESX-DNA complex almost entirely, 100 nM distamycin had no effect on complex formation (Figure 2A, lane 2 and Figure 2B, lane 6). A quantitation of the data in Figure 2C indicated that 2.2 nM of PA-2E and 500 nM of distamycin are needed to inhibit complex formation by 50% (IC_{50}) (Table 1). The activity of individual drugs for inhibiting TF-DNA complexes is also expressed as r values, the molar ratio of drug to DNA base pairs (Table 1). Expressing drug concentration as r value allows for comparison of individual drugs in assay systems which use vastly different DNAs as gel mobility shift assay of TF-DNA complex formation and *in vitro* transcription.

Effects of Polyamides on ESX-DNA Complex Formation. Several PAs were synthesized to target DNA sequences around the consensus core-binding site of ESX. PA-1E and -2E recognize DNA sequences upstream and downstream of the core GGAA binding site, respectively, while PE-3E recognized a modified version of the PA-2E DNA binding site (see Figure 1). Our initial data showed that PA-2E was about 250-fold more potent than distamycin at inhibiting the ESX-DNA

complex (Table 1). We wished to compare how these PAs that recognize different parts of ESX binding site could influence ESX-DNA complex formation. Gel mobility shift assays were used to test the activity of polyamides. Results from gel mobility shift assays shown in Figure 3 indicated that PA-1E and -2E were quite similar in their ability to inhibit the complex formation. IC_{50} for PA-1E was 5 nM which is ~ 2-fold greater than the IC_{50} for PA-2E. In comparison, PA-3E was a relatively weaker inhibitor of ESX-DNA complex and required a 9-fold higher drug concentration (18 nM) compared to PA-2E (2.2 nM) to inhibit 50% complex formation (Figure 3; Table 1).

Equilibrium Study. The evaluation of PAs designed to inhibit the ESX-DNA complex was measured under equilibrium conditions. We and others have noted that, for certain combinations of drugs and TFs, the conditions to reach equilibrium require greater concentrations of drug when drug is given to a preformed complex (reverse) rather than prior to the TF (standard) (5,8). In such cases, it is possible that drugs which more directly compete for the TF DNA binding domain require longer time or greater concentrations to establish equilibrium in regard to complex inhibition.

Our data indicated that PA-1E throughout a wide concentration range established an equilibrium under similar conditions for inhibiting complex formation in both standard and reverse reactions (Figure 4A). For example, at 10 nM drug concentration nearly the same level of complex was formed in both reactions within 30 min (Figure 4A). In comparison, PA-2E which inhibited complex formation under equilibrium conditions required 10-fold higher drug (100 nM) concentration in the reverse assay (Figure 4B). To demonstrate that a longer incubation time was required for PA-2E to reach equilibrium in comparison with PA-1E, a time course assay with 10 nM of PA-2E was performed under a reverse condition. The result indicated that percentage of inhibition of ESX-DNA complex by PA-2E increased with longer incubation time and $\geq$ 4h was needed to reach equilibrium conditions (Figure 4C).

Effects of polyamides on AP-2-DNA Complex Formation. Previous studies showed that the AP-2 was involved in the overexpression of the HER2/*neu* gene and footprinting analysis revealed that AP-2 competed with Sp1 binding for the GC-box on the HER2/*neu* gene promoter (26). We demonstrated that AP-2 recognized a GC-rich sequence just upstream of the ESX binding-site at

the HER2/*neu* promoter. The binding of AP-2 to the GC-rich sequences in TA5-oligo (Figure 1) was confirmed using gel mobility shift assays in the absence or presence of antibodies. The data indicated that AP-2 bound to the TA5-oligo under our assay condition and that specific antibodies against AP-2 removed the protein-DNA complex (Figure 5A, lane 3), while non-specific antibodies or normal immunoglobulin had no effect on complex formation (Figure 5A, lanes 4 & 5).

Since we have demonstrated that AP-2 interacts with GC-rich sequences which are a few base pairs upstream of the ESX binding-site, it was of interest to know whether the PAs that were originally designed for targeting the ESX binding site would affect the DNA binding of AP-2. Gel mobility shift assays were used to address this issue, and the data in Figure 6B showed that PA-1E was capable of inhibiting DNA binding of AP-2 in a concentration-dependent manner with an IC_{50} of ~ 48 nM (Figure 5B; Table 1). In contrast, PA-2E was unable to block complex formation even at the highest drug concentration (100 nM) tested (Figure 5C) (drug concentration greater than 100 nM caused smearing of the DNA from the gel under our assay condition). The pattern of inhibition of the AP-2-DNA complex by distamycin was similar to that of PA-2E in that micromolar drug concentration was required for both drugs to inhibit AP-2-DNA complex formation at certain levels (Figure 5D; Table 1). These drugs are more efficient at inhibiting the complex formation of ESX than AP-2. PA-2E is clearly the most specific inhibitor of ESX.

Effect of PAs on the *In Vitro* Transcription. To determine whether the effects of PAs on the TF-DNA complex formation imply that PAs are able to influence biological function. *In vitro* transcription assays were carried out. The plasmid DNA (RO6) linearized with *Sph*I was used as a template and SKBR-3 nuclear extracts as transcription machinery resulting in a ~760 base transcript. The first experiment performed was to incubate drugs with DNA template prior to the addition of nuclear extracts and nucleotides (standard *in vitro* transcription). A representative gel is shown in Figure 6A demonstrates the ability of PA-2E to efficiently block synthesis of the 760 base transcript in a concentration-dependent manner. Five μM PA-2E inhibited transcript by 95% while 1 uM blocked transcript less than 50% comparing with the untreated control. There was of the production of partial transcripts were observed when higher drug concentrations were used (Figure 6A, lanes 3 & 4). Quantitation of the data for the inhibition of *in vitro* transcription by

PA-1E, -2E, -3E, and distamycin is shown in Figure 6B. The order of potency at inhibiting transcription from the HER2/*neu* promoter by these drugs was somewhat different from that for inhibiting TF-DNA complex formation: PA-2E > -3E > -1E > distamycin. The IC_{50} for individual drugs are listed in Table 2. Drug concentrations of 1.4 μM for PA-2E, 3.2 μM for PA-1E, 2.4 μM for PA-3E, and 7.4 μM for distamycin were required to inhibit transcription by 50%. Data is also expressed as r values so that the activities of individual drugs for their ability to inhibit transcription can be compared with inhibition of TF-DNA complexes.

Earlier studies demonstrated differences between the ability of PA-1E and -2E to inhibit ESX complexes when drug was given before or after the formation of the ESX-DNA complex (Figure 4). To determine whether the order of addition of drugs influences the activity of these drugs for inhibiting transcription, nuclear extracts were allowed to interact with DNA prior to the addition of drugs. It was not surprisingly that the activity of both drugs was reduced when tested in a reverse versus a standard assay (Figure 7). For example, PA-2E at drug concentrations of 1.4 and 4.2 μM were required for inhibiting transcription by 50% in the standard and reverse assays, respectively (Figure 7A). In the case of PA-1E, a two-fold higher drug concentration was needed to inhibit a reverse transcription (6.4 μM) than to inhibit a standard transcription (3.2 μM) (Figure 7B).

Time course. The pattern of transcription in the presence of drugs was examined by a time course. Previous study showed that the amount of transcript was relatively increased along with the reaction time when a constant drug concentration that inhibited ≤ 50% of transcript was used suggesting that drugs partially occupied the promoter allowing some initiation of transcription (15). In this experiment, the most potent inhibitor PA-2E was used to examine how PAs interfered with transcription from the HER2/*neu* promoter. Our data revealed that a constant level of inhibition of transcription in the presence of PA-2E was throughout the examined time points (i.e., 10, 30, and 60 min). In comparison, inhibition of transcript by distamycin was similar to the previous finding that transcript was increased relative to the time of incubation. Difference between PAs and distamycin in the pattern of inhibition of transcripts is possibly resulted from the

high affinity of PAs for the DNA in which drugs always associate with DNA to interfere with the initiation of transcription.

Discussion

In this study, we examined the ability of sequence-specific PAs designed to inhibit TF-DNA complex formation and associated transcription from the Her2/*neu* promoter. Several PAs were synthesized that recognized different sequences associated with the ESX consensus DNA binding site within the Her2/*neu* promoter. The ability of these compounds and distamycin to inhibit ESX-DNA complexes was measured by mobility shift assays. Based upon footprint data, PA-2E which was the most effective inhibitor, binds downstream and to the ESX core consensus site (GGA) with an association constant of $1.5 \cdot 10^{10}$/M. Certainly, the recognition of a common DNA sequence between the drug and TF contribute to the inhibitory ability of PAs. Similarly, the general TF TBP which binds AT rich sequences in the DNA minor groove is strongly inhibited by distamycin, a DNA minor groove binding drug (8). Likewise, drugs which prefer GC rich DNA binding sites like mithramycin and nogalamycin are strong inhibitors of transcription factors like c-myc and EGR which bind to GC rich DNA promoters (5,7).

Comparison of the abilities of the three PAs with that of distamycin to inhibit ESX-DNA complexes reveals that the potency of the former compounds are one to two orders of magnitude greater. Since both distamycin and PA-2E would bind within site 2 (see Figure 1), the very high affinity constant of the latter likely contributes to differences in activity. It is also possible that the ability of PAs to bind in a side by side fashion within the DNA minor groove, with the resulting distortion of the major groove, contributes to their strong activity. Since ETS family members utilize contacts with both DNA grooves for optimal binding to promoters, some of the inhibition can also be attributed to steric effects that restrict ESX access to the DNA (27-29). In addition, minor groove binding drugs are capable of altering the DNA grooves by widening the minor groove and narrowing the major groove (30). As a consequence, these drugs also have the ability to inhibit TF-DNA complexes that only utilize the major groove. For example, the GC specific DNA minor groove binding agents chromomycin A, very effectively inhibits EGR complexes with its GC rich DNA consensus binding site in the DNA major groove (5). Also, the AT specific DNA minor groove binding drug distamycin, inhibits homeodomain complexes with its AT rich DNA consensus binding site (31). The relatively lower activity of PA-3E, which binds the same site as

PA-2E may be attributed to its somewhat lower DNA binding constant. On the other hand, PA-1E which binds upstream of the GGA core consensus sequence was nearly as active as PA-2E.

The potential for specific targeting of TFs with PAs is exemplified by comparing drug ability to inhibit ESX and AP-2-DNA complexes. PA-2E the strongest inhibitor to the ESX-DNA complex was nearly inactive inhibitor of AP-2 binding to a GC rich region adjacent to site 1 (Figure 5C and Table 1). Similarly, distamycin which binds like PA-2E to site 2 showed a relative weak ability to inhibit AP-2-DNA binding (Fig. 5D and Table 1). On the over hand, PA-1E which binds site 1 was moderately active as an inhibitor of AP-2-DNA complexes (Figure 6B and Table 1). Localized drug targeting has been seen with the Herpes Virus Latency Associated Transcript promoter where drugs such as nogalomycin were found to disrupt the binding of EGR to its GC rich consensus site, while not blocking the binding of TBP to adjacent AT rich site (32).

Another property of PAs as inhibitors of ESX-DNA complexes was noted in the differences between the inhibition profiles of PA-1E and PA-2E when analysis was carried out where drug was added either before (standard) or after (reverse) the TF was allowed to complex with the DNA. In the case of PA-1E, the overlapping curves between inhibition of complex under both standard and reverse conditions demonstrate that equilibrium was reached within the 30 min incubation time (Figure 4A). In contrast, PA-2E under reverse conditions required significantly higher drug concentrations 100 nM) to reach inhibition levels comparable to those found in the standard assay (10 nM) (Fig. 4B). Alternatively, when the time to reach equilibrium was extended in the reverse assay to 240 minutes, 10nM PA-2E inhibited the complex at comparable to the level found in the standard assay (Fig. 4C).

We and others have observed that drugs which bind to DNA near or at the TF consensus binding site typically require more time (or more drug) to reach an equilibrium value for complex inhibition when given after the complex is formed in comparison to adding drug prior to the addition of the TF (5,8). The findings with PA-1E and PA-2E could suggest that drugs targeting the site 1 region upsteam of the ESX core consensus binding site have less interference from the TF coming on and off the DNA than do the drug targeting the downstream site 2 region.

PAs that effectively inhibited ESX-DNA complexes were also found to be highly efficient at inhibiting in cell free transcription driven by the Her2/*neu* promoter. While all three compounds were effective inhibitors, PA-2E, which was the most active inhibitor of the ESX complex, was also the most active transcription inhibitor (Figure 6). In contrast, PA-3E was a somewhat (50%) more active transcription inhibitor than PA-1E, yet it was noticeably less active (~ 3-fold) at inhibiting ESX-DNA complexes. One possible explanation is the fact that transcription analysis is carried out in assays that contain nuclear proteins which interact with the promoter and might alter the ability of a drug to disrupt protein promoter complexes. A similar observation was found when we studied mitoxantrone and distamycin as inhibitors of TF-DNA complex formation and *in vitro* transcription on the hamster dihydrofolate reductase gene promoter (15). The results indicated that mitoxantrone was about 3-fold stronger than distamycin at inhibiting E2F1-DNA complex formation while both drugs inhibited transcription similarly.

Our data showed that the order of addition of drugs to reactions that contained DNA alone or a preformed TF-DNA complex (standard or reverse) could influence the drug activity in gel mobility shift assays (Figure 4). Transcription analysis was also carried out under conditions where drug was added after the DNA template had been incubated with the nuclear extracts. As predicted, PA-1E activity was diminished indicating that once proteins had been loaded onto the promoter, the drug was less able to block transcription. The same effect was observed for PA-2E except the differences were even greater (Figure 7). Since PA-2E was also less effective at inhibiting ESX-DNA complexes if added after the complex formed, may account for the greater decrease in its activity in the reverse transcription assay. Together, the differences in activities of PA-1E and –2E to interfere with TF-DNA complex and transcription are probably not only due to the interaction of proteins and DNA but also to the natures of individual PAs.

To this end, we have examined the effects of PAs on the inhibition of TF-DNA complex formation on the HER2/*neu* promoter and subsequently tested for their ability to interfere with gene transcription. To determine whether there is a correlation of drug activity between these two assay systems, drug concentrations needed to inhibit complex formation or transcription were

expressed as r values to adjust for differences between the two assays in terms of DNA content. The fact that the r values were lower for all tested compounds when they were used in transcription assays comparing with mobility shift assays suggested that drug preference for other sequences within the plasmid DNA did not diminish specificity for the promoter region (Tables 1 & 2). However, the results from this study were not enough to elucidate why the r value of distamycin was dramatically reduced in transcription assay.

This study demonstrates the value of polyamides as a means to selectively target TF DNA binding domains. These sequence-specific compounds designed for high affinity binding to the targeted DNA binding domains of selected TFs are potent inhibitors of complex formation and transcription. By examining their mode of action in detail under cell free-conditions, we were able to uncover differences in drug activities that are dependent upon which part of the consensus binding site was being targeted. This information can be utilized to further improve drug specificity and effectiveness as selective transcription inhibitors. Studies are underway to evaluate the effectiveness of these drugs as transcription inhibitors in cells.

Legends

Figure 1. Sequence of TA5-oligo and structure of polyamides. A bold sequence of GGAA is the core binding site of ESX; an underline sequence is the putative AP-2 binding site; sequences labeled as 1 and 2 are targeting sequences of PA-1E, -2E, and –3E, respectively (A). Structures of PA-1E, -2E, and –3E are depicted in (B).

Figure 2. Effects of PA-2E and distamycin on ESX-DNA complex formation. A representative gel mobility shift assay in the presence of PA-2E was used to evaluate the ability of polyamides to inhibit ESX binding to the TA5-oligo that was derived from the HER2 promoter (A). DNA and drug were incubated for 30 min at room temperature followed by the addition of ESX and a subsequent 30 min incubation. Samples were then loaded onto a 5% native polyacrylamide gel. Lane 1, control of ESX-DNA; lanes 2-6, samples in the presence of PA-2E at concentrations of 100, 10, 1, 0.5, and 0.1 nM, respectively; lane 7, control of free DNA. A gel mobility shift assay in the presence of distamycin was carried out under the same assay condition as described for PA-2E (B). Lane 1, control of ESX-DNA; lanes 2-6, samples in the presence of distamycin at indicated concentration of 10 5, 2, 1, and 0.1 μM, respectively; lane 7, control of free DNA. Quantitation of ESX-DNA complex formation in the presence of PA-2E and distamycin was plotted in (C). The percent inhibition of ESX-DNA complex formation by drugs was measured by comparing drug-treated sample with an untreated control. PA-2E (●) and distamycin (▼) at the indicated concentrations were incubated with the oligo prior to the addition of ESX protein. After electrophoresis, a dried gel was exposed to a Kodak film followed by quantitation using a densitometer. The data was the mean of at least three separated experiments (± SD).

Figure 3. Inhibition of ESX-DNA complex by polyamides. The ability of polyamides to inhibit the DNA binding of ESX was examined by mobility shift assays. Experiments were performed as described in Figure 3A. DNA and polyamides, PA-1E (●), -2E (▼), and -3E (■) were incubated for 30 min at room temperature followed by addition of ESX, respectively. Quantitation of the data was the mean of three separated experiments (± SD).

Figure 4. Equilibrium study. Reverse mobility shift assays were performed to evaluate whether drug inhibition of ESX-DNA complex reached equilibrium under conditions similar to the standard assay. Experimentally, PA-1E (A) and –2E (B) at indicated concentrations were added to a preformed complex of ESX and DNA, respectively. The drug inhibition of complex formation in a reverse assay (◆) was compared with that in a standard assay (■). The data shown was the mean of at least three separated experiments (± SD). A time course assay was used to estimate the time required for PA-2E to reach the equilibrium in terms of inhibition of ESX-DNA complex formation (C). ESX and DNA were incubated at room temperature for 30 min followed by addition of drug for 240, 120, 60, 30, and 15 min, respectively. The assay was repeated several times and the results were expressed as mean ± SD.

Figure 5. Effects of drugs on complex formation of AP-2. Confirmation of DNA binding of AP-2 in the presence of antibodies was performed using a mobility shift assay (A). Experimentally, AP-2 and antibodies were incubated at room temperature for 10 min prior to the addition of ^{32}P-labeled TA5-oligo. Lane 1, control of free DNA; lane 2, AP-2-DNA complex; lanes 3-5; reactions with antibodies of A (AP-2), S (Sp1), and N (normal immunoglobulins), respectively. Inhibition of the AP-2-DNA complex (▼) was compared with the formation of ESX-DNA complex (●) in the presence of PA-1E (B), -2E (C), or distamycin (D), respectively. Each experiment was repeated several times with duplicate samples. The results were expressed as mean ± SD.

Figure 6. Effects of drugs on *in vitro* transcription. Activities of drugs in inhibiting gene transcription were compared using a standard *in vitro* transcription assay. PA-2E at the indicated concentrations were incubated with a restriction enzyme *Sph*I-digested DNA template (RO6) at 30 °C for 30 min followed by the addition of SKBR-3 nuclear extracts for 15 min and nucleotides. Transcription was allowed to proceed for 60 min at 30 °C and a ~ 760 base of transcript was formed. A representative *in vitro* transcription experiment in the presence of PA-2E is shown in (A). Lane 1, untreated control; lanes 2-4, in vitro transcription treated with PA-2E at concentrations of 1, 2.5, and 5 μM, respectively; lane 5, RNA marker. Tc indicates a760 base-pair transcript and IC indicates an internal control. Activities of drugs, PA-1E (●), -2E (▼), -3E (■), and distamycin (◆) were presented as percentage of inhibition of transcription by comparing drug-

treated samples with an untreated control (B). The transcription activity of individual samples was normalized to an internal control. The results were the mean of several experiments ± SD.

Figure 7. Effects of drugs on reverse transcription. The order of addition of drugs was examined using a reverse transcription study. The experimental procedure was similar to that described in Figure 7A except that DNA template was incubated with SKBR-3 nuclear extracts for 15 min before adding drugs and nucleotides. PA-2E (A) and –1E (B) were tested for their ability to interfere with transcription. The percent inhibition of transcription (▼) by individual drugs were compared with that of transcription carried out under standard assay condition (●). The results were expressed as mean of several experiments ± SD.

Figure 8. *In vitro* transcription time course in the presence of drugs. The time course of *in vitro* transcription was performed as described in Figure 8 except that the transcription reaction was stopped at time points of 5, 10, 30 and 60 min. Quantification of in vitro transcription in the presence of PA-2E (●) and distamycin (▼) compared with non-drug-treated control. The results represent several experiments (mean ± SD).

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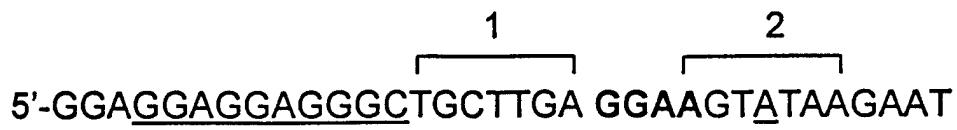
A



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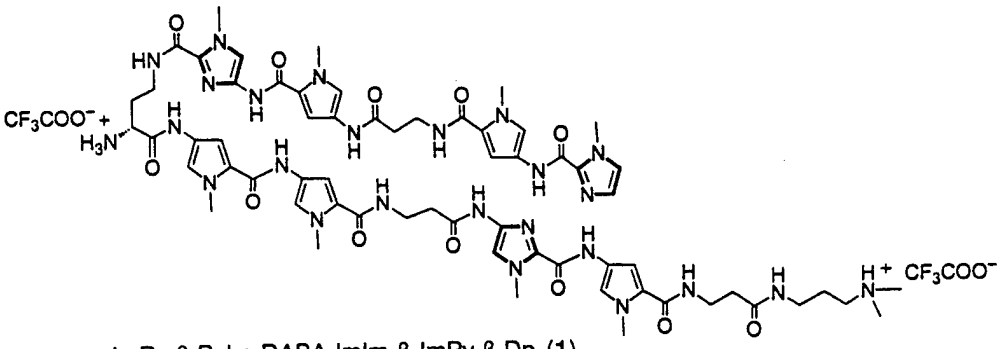

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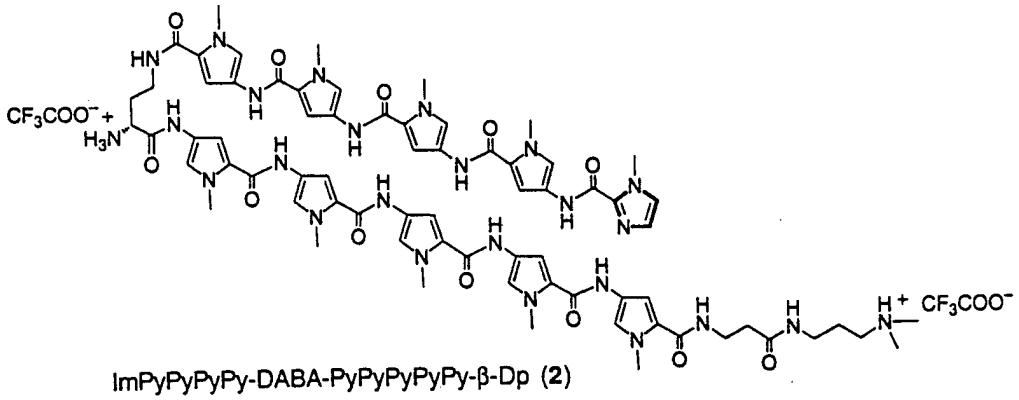
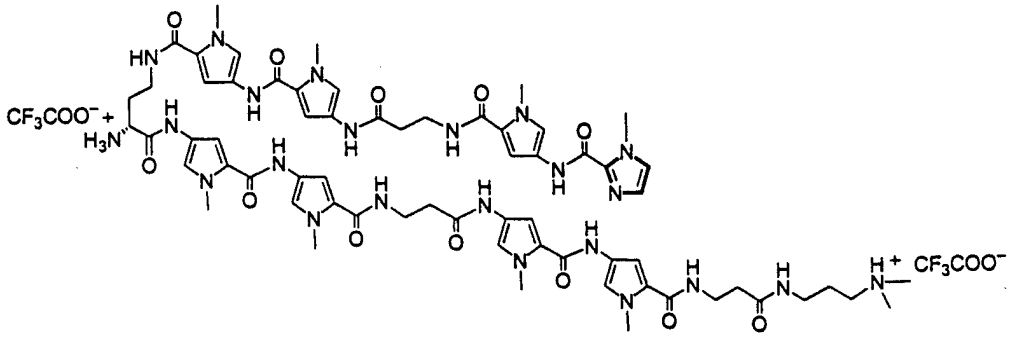
ε = 53,200 $M^{-1}cm^{-1}$ at 300 nm



ImPyPyPyPy-DABA-PyPyPyPyPy-β-Dp (2)

ε = 70,200 $M^{-1}cm^{-1}$ at 300 nm



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ε = 53,400 $M^{-1}cm^{-1}$ at 300 nm

Figure 1

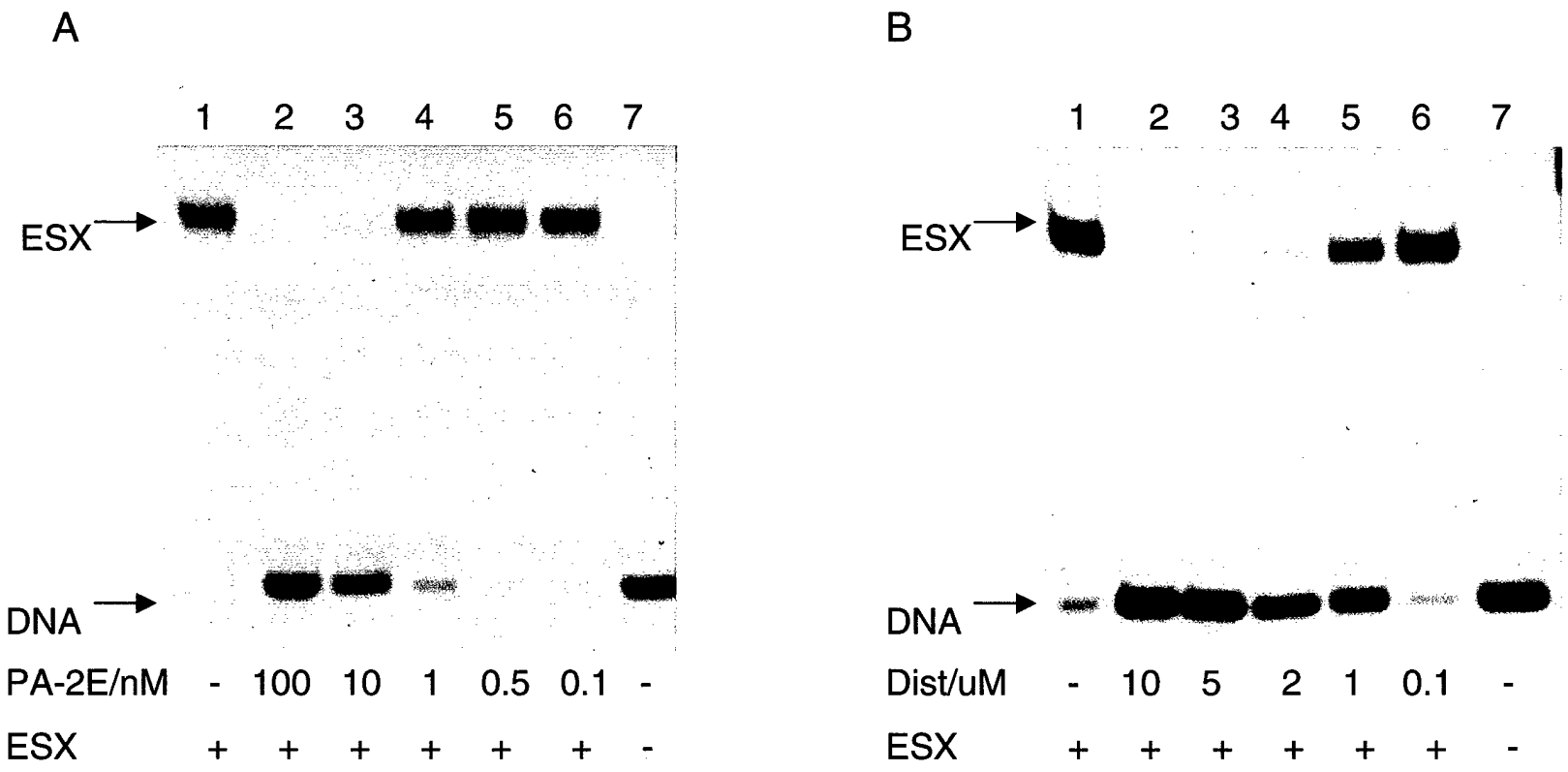
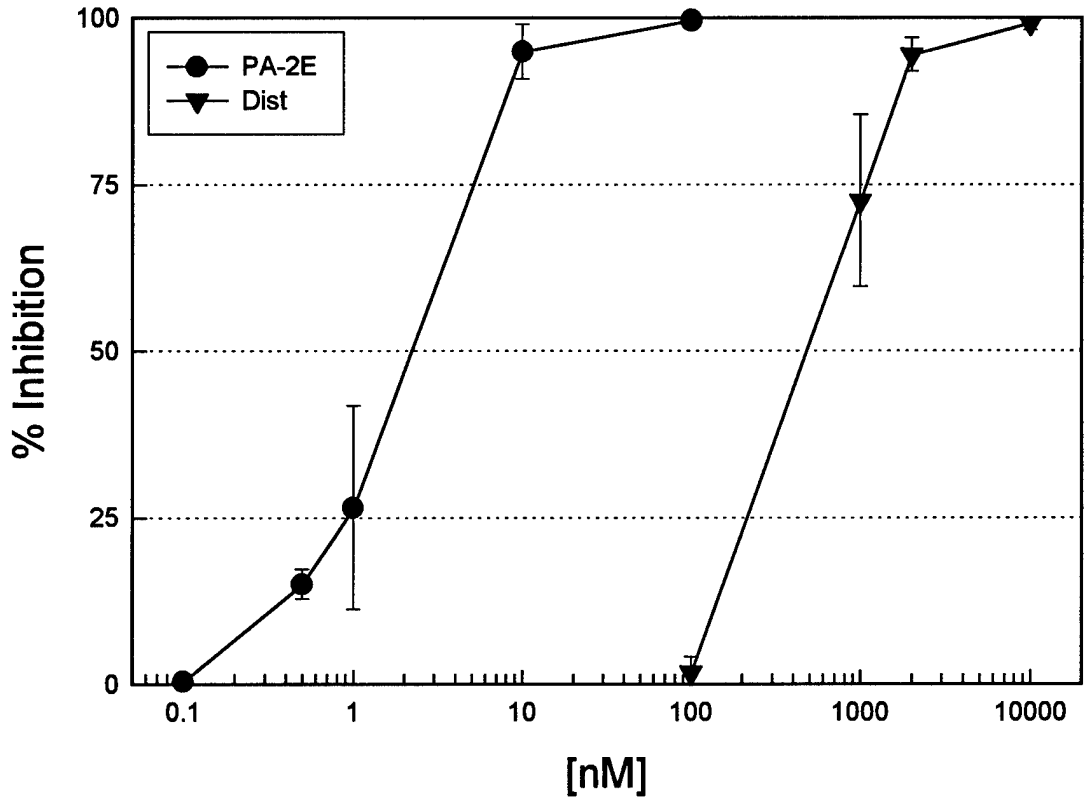


Figure 2, Chiang et al

C



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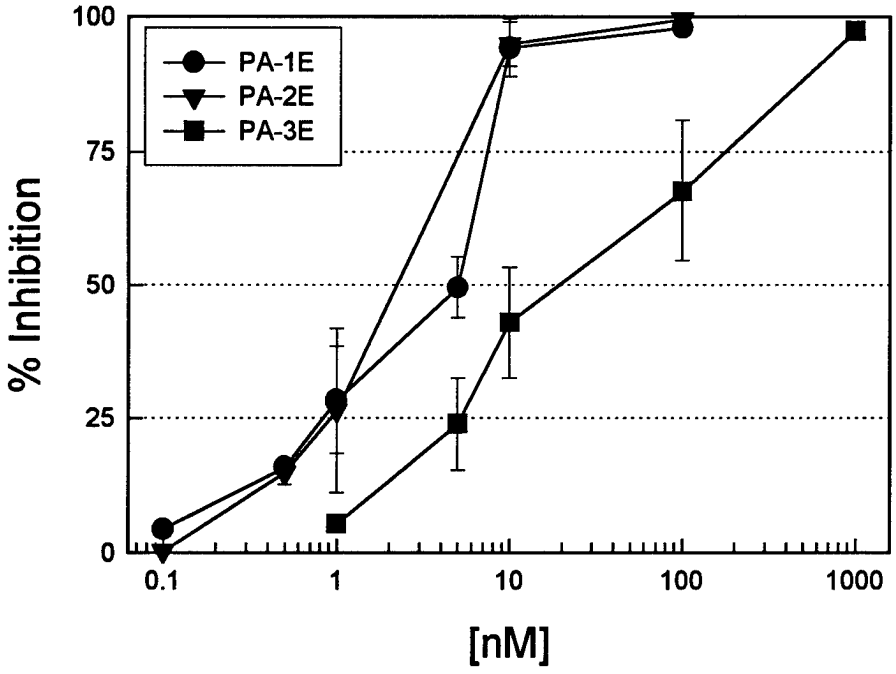

Figure 3, Chiang et al

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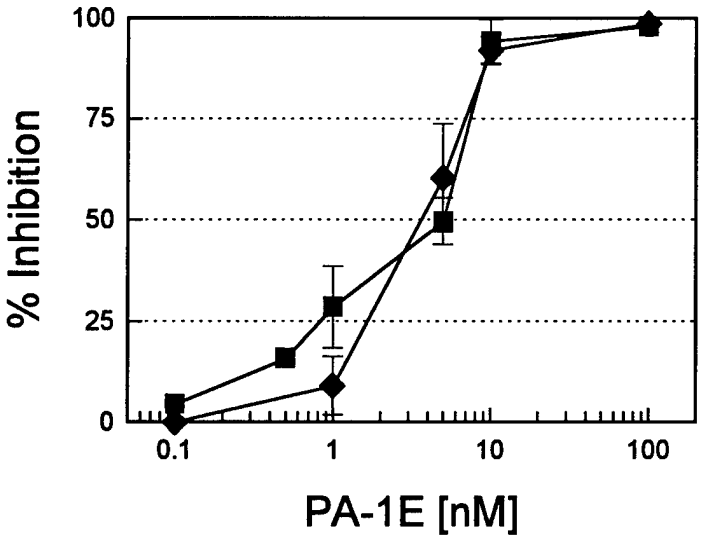

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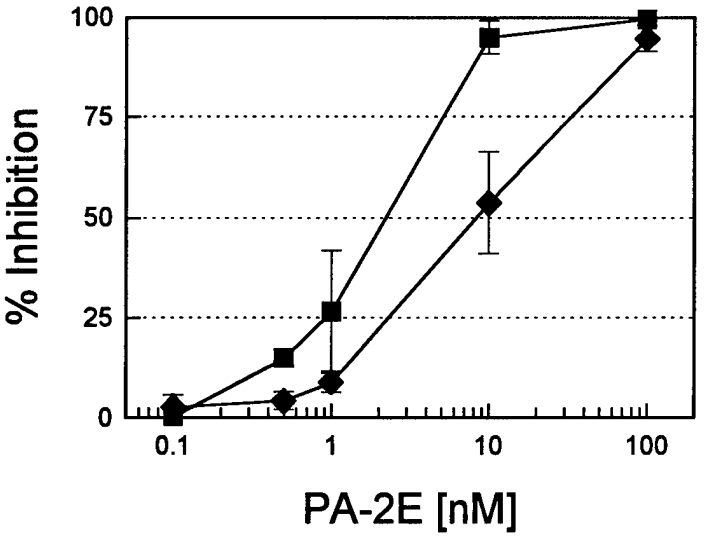

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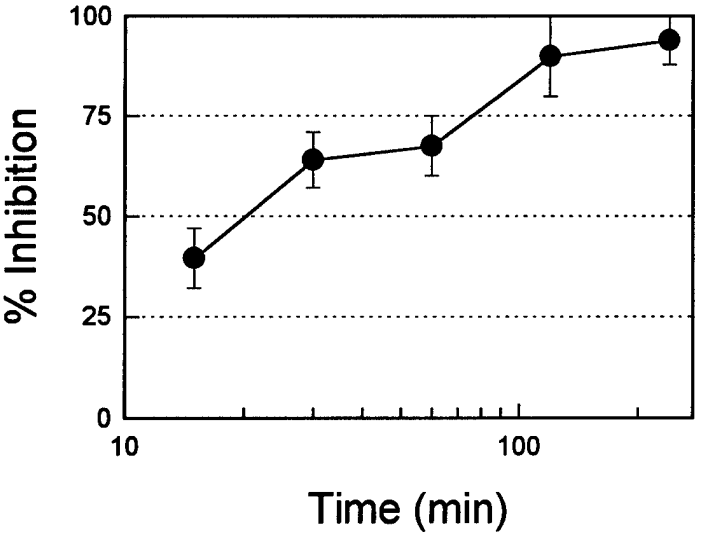

Figure 4, Chiang et al

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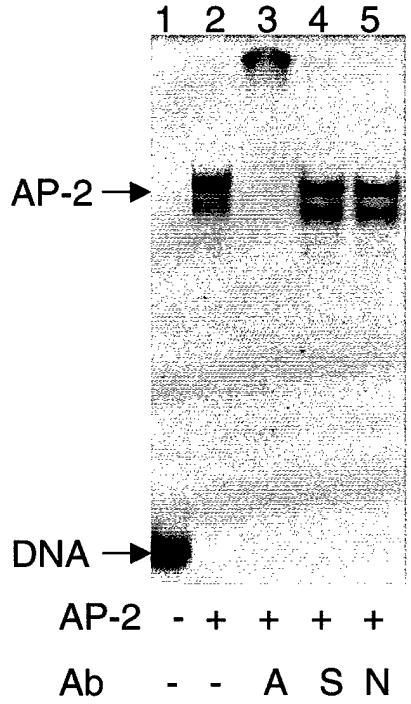

Figure 5, Chiang et al

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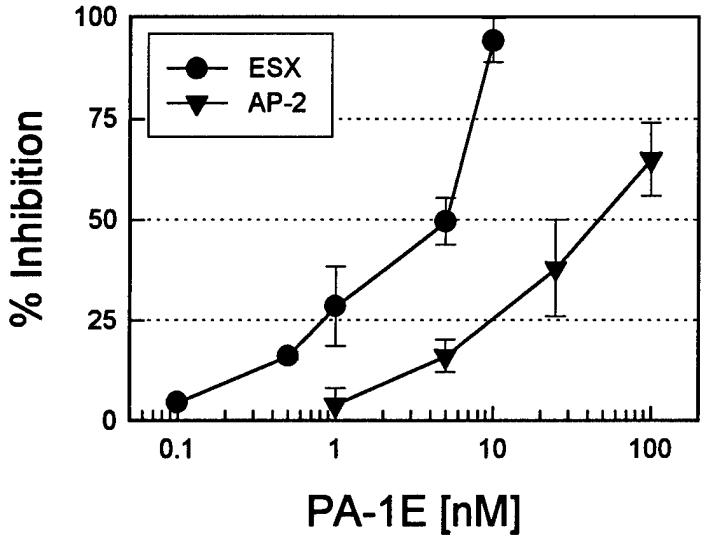

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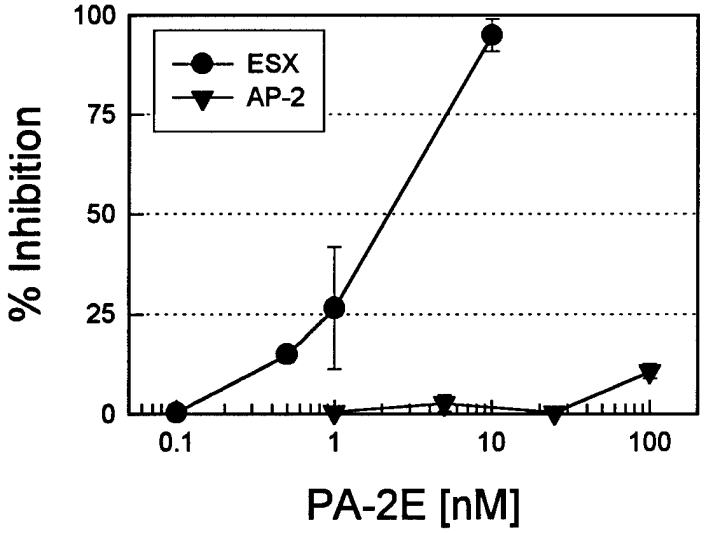

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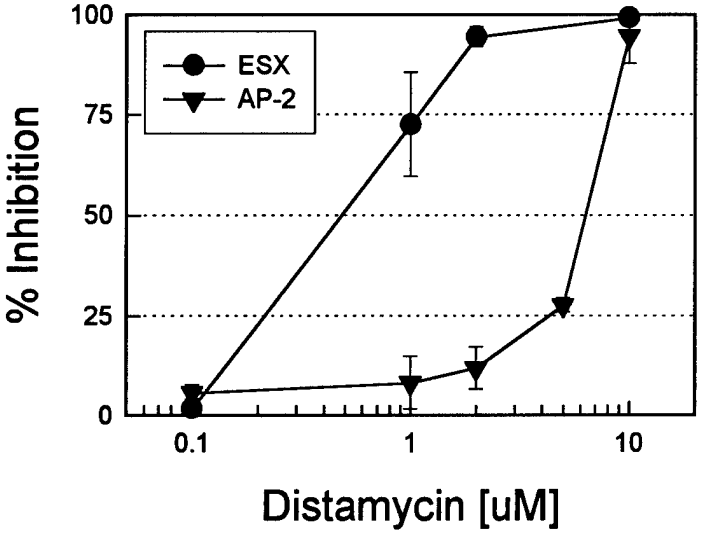

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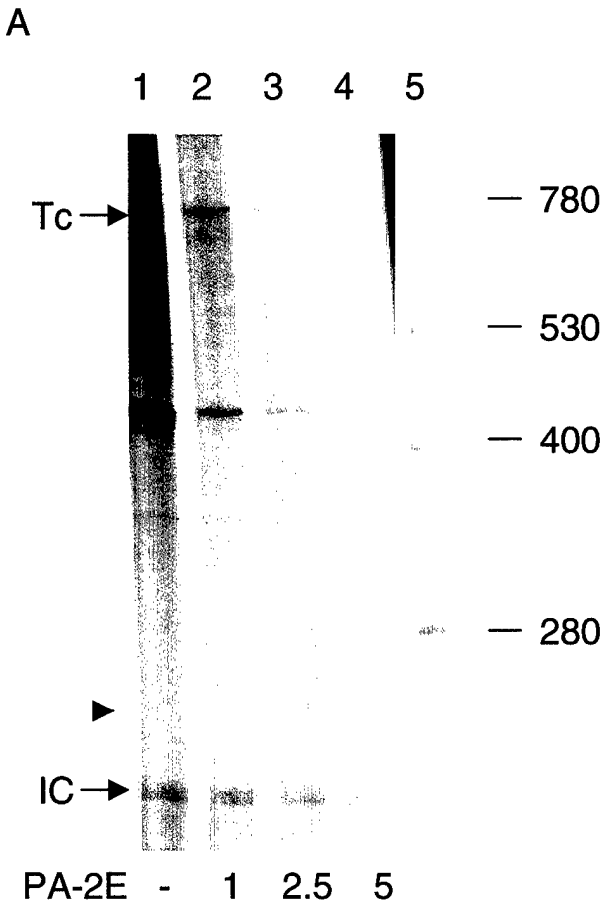

Figure 6, Chiang et al

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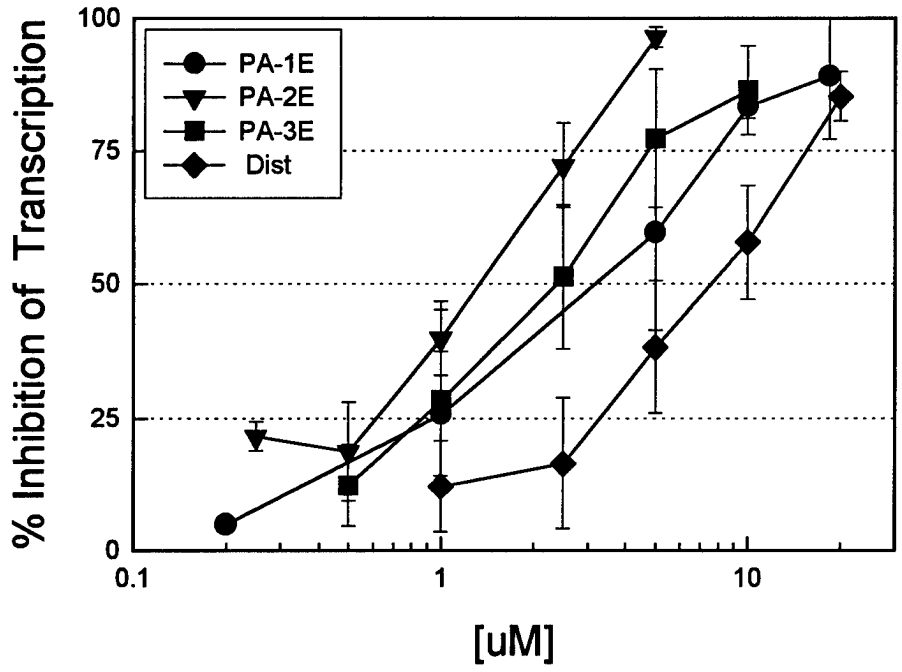

Figure 6 (continued)

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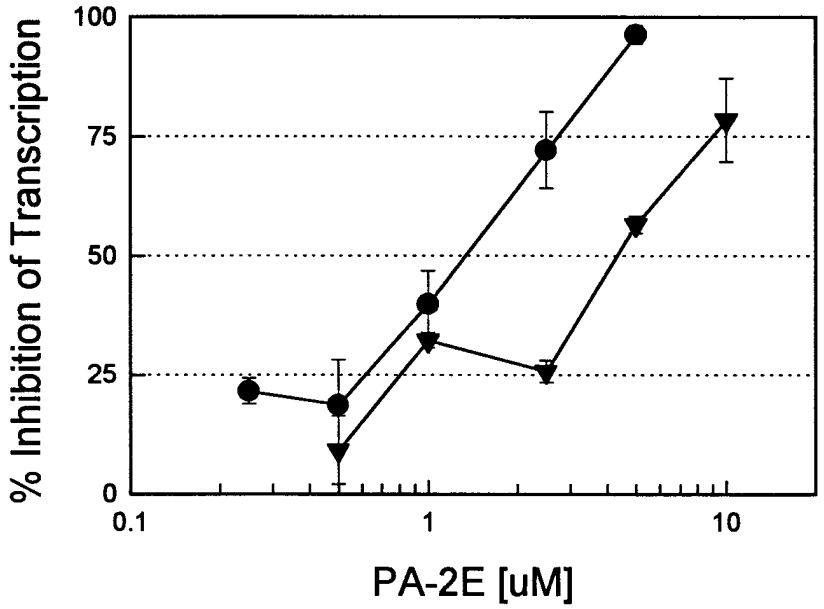

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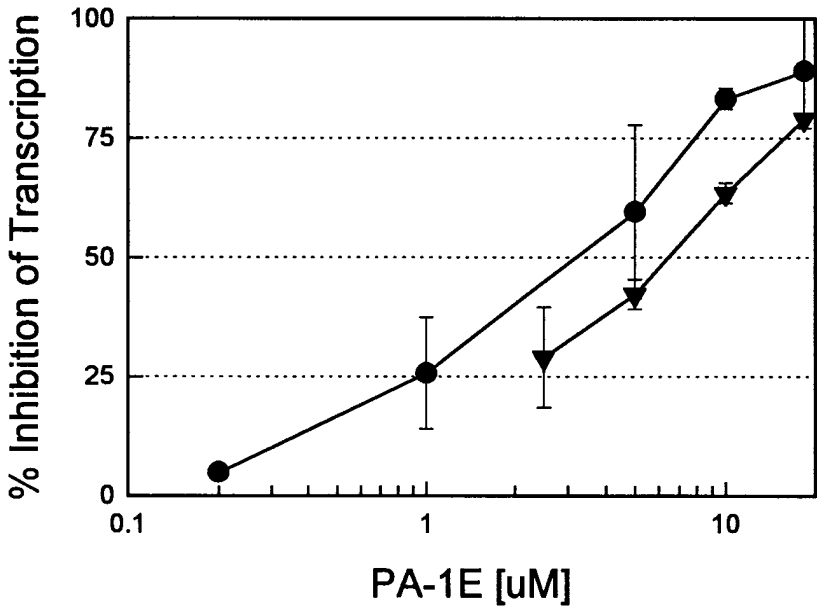

Figure 7, Chiang et al

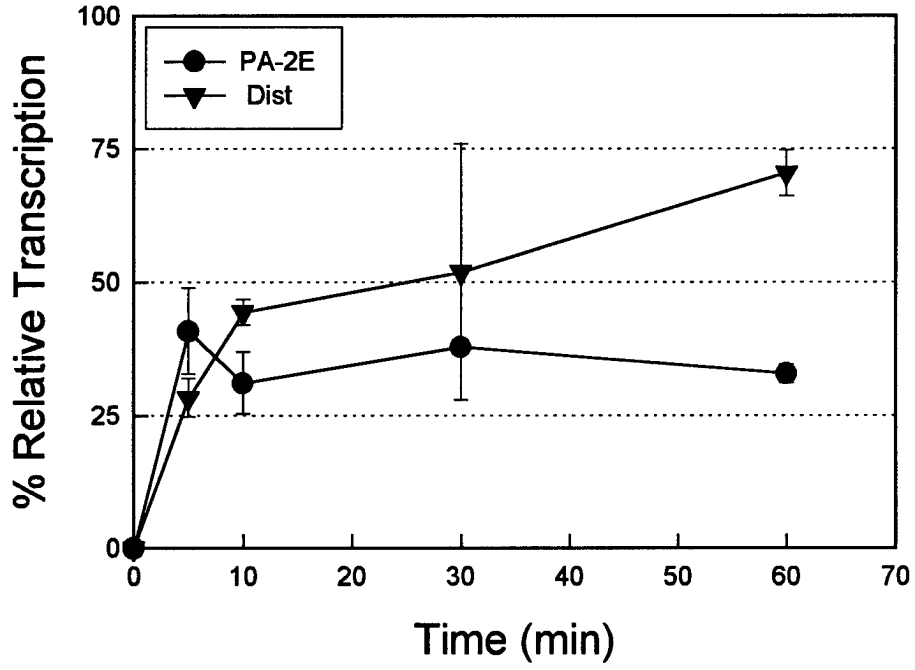

Figure 8, Chiang et al

Table 1. Drug Effects on TF/DNA Complex Formation

Drug	TF	IC_{50} [nM]	r value[1]
PA-1E	ESX	5	0.16
	AP-2	48	1.55
PA-2E	ESX	2.2	0.07
	AP-2	> 100	ND[2]
PA-3E	ESX	18	0.58
	Ap-2	ND	ND
Distamycin	ESX	500	16.1
	AP-2	6000	193.5

[1] r value = the molar ratio of drug to DNA base pairs.
[2] ND, not done.

Table 2. Drug Effects on the *In Vitro* Transcription

	IC_{50}	
Drug	[μM]	r value
PA-1E	3.2	0.02
PA-2E	1.4	0.009
PA-3E	2.4	0.015
Distamycin	7.4	0.05